EXHIBIT 11

0022-1767/93/15011-5185$02.00/0
The Journal of Immunology

Vol 150, 5185–5194, No. 11, June 1, 1993
Printed in U.S.A.

Induction of Diabetes Is Influenced by the Infectious Virus and Local Expression of MHC Class I and Tumor Necrosis Factor-α[1]

Pamela S. Ohashi,[2,3]* Stephan Oehen,[4]* Peter Aichele,* Hanspeter Pircher,* Bernhard Odermatt,* Pedro Herrera,† Yasunori Higuchi,[5]‡ Kurt Buerki,§ Hans Hengartner,* and Rolf M. Zinkernagel*

*Department of Pathology, Institute of Experimental Immunology, University of Zurich, 8091 Zurich, Switzerland, *Department of Pathology and †Institute of Histology and Embryology, Centre Medical Universitaire, University of Geneva, 1211 Geneva 4, Switzerland, and §Preclinical Research, Sandoz Pharma Ltd., 4002 Basel Switzerland

ABSTRACT. To study self reactivity, a transgenic mouse model has been established in which the lymphocytic choriomeningitis virus (LCMV) glycoprotein (gp) is expressed in the β-islet cells of the pancreas (rat insulin promoter (RIP)-gp). These mice (H-2^b) do not spontaneously develop diabetes; however, infection with the LCMV strain WE rapidly induces hyperglycemia. In this study, comparative analysis of H-2^k RIP-gp-transgenic animals demonstrated that the haplotype influences the incidence and kinetics of diabetes and alters the requirement for the CD4$^+$ T cell subset. This study also showed that the properties of the virus expressing the self target Ag determined whether hyperglycemia occurred in RIP-gp-transgenic mice. Various LCMV strains were able to induce diabetes in RIP-gp-transgenic animals, whereas infection with a recombinant vaccinia virus expressing LCMV-gp (vacc-gp) did not induce diabetes. However, vacc-gp could induce diabetes in double (RIP-gp/TCR)-transgenic mice, where the majority of CD8$^+$ T cells expressed a receptor specific for LCMV-gp, suggesting that a critical number of self-reactive T cells must be activated to induce disease. Noteably, histologic analysis of pancreata taken various days after LCMV or vacc-gp infections indicated that induction of diabetes coincided with an increase in MHC class I expression on the islets of Langerhans. Additional studies with vacc-gp were done to determine other factors that possibly enhance autoimmune attack. Transgenic mice expressing both LCMV-gp and TNF-α under the control of the RIP were infected with vacc-gp, and 50% of RIP-gp/TNF-α-transgenic animals became hyperglycemic. These data suggest that the increased local lymphocyte traffic as a result of TNF-α expression attracts activated gp-specific T cells, enhancing the possibility of hyperglycemia. Collectively, these results demonstrate that the induction of diabetes in this model is influenced by the MHC haplotype, the infectious agent, TNF-α expression, the level of MHC class I expression, and the induction of a threshold number of self-reactive CTL. *Journal of Immunology*, 1993, 150: 5185.

Received for publication November 12, 1992. Accepted for publication February 22, 1993.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Swiss National Fund (grants 31–32195.91 and 31–32179.91). P. S. O. is supported by the Medical Research Council of Canada.

[2] Address correspondence and reprint requests to Pamela S. Ohashi, Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9.

[3] Present address: Ontario Cancer Institute, Department of Medical Biophysics, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, Canada M4X 1K9.

[4] Present address: Department of Biology, University of California, San Diego, La Jolla, CA 92093–0063.

[5] Present address: Department of Pathology, Medical College of Oita, Oita 879–56, Japan.

Table I
Transgenic mice used in this study

Transgenic Line	Promoter	Transgene	Properties
RIP-gp(Bln)	RIP II	LCMV-gp	Expression of LCMV-gp on β-islet cells in the pancreas
RIP-gp(Brx)	RIP II	LCMV-gp	Expression of LCMV-gp on β-islet cells in the pancreas
RIP-TNF	RIP II	TNF-α	Local expression of TNF-α, resulting in heavy lymphocytic infiltration
327	H-2K^b, Ig enhancer	P14 TCR α- and β-chains	Expression of receptors specific for LCMV-gp plus H-2D^b on 95% of CD8$^+$ T cells
318	H-2K^b, Ig enhancer	P14 TCR α- and β-chains	Expression of receptors specific for LCMV-gp plus H-2D^b on 50% CD8$^+$ T cells

Autoreactive T cells have the potential to mediate immunopathologic destruction of target tissues and have been implicated in many autoimmune disease processes (1–3). Several studies have shown that potentially self-reactive T cells may be rendered nonfunctional by T cell tolerance to self Ag expressed in the thymus or by extrathymic mechanisms of peripheral tolerance (4–9). However, other models are consistent with the idea that T cell tolerance to certain Ag does not occur because the self Ag is neither tolerogenic nor immunogenic and therefore may be ignored by the immune system (10–16). Several questions arise if T cells have not been tolerized to all self proteins. Why does autoimmune disease not occur more frequently and what processes are important in triggering these potentially autoreactive cells? We have addressed some of these issues by using a virus Ag-transgenic model for diabetes.

Transgenic mice expressing LCMV[6]-gp in the β-islet cells of the pancreas have been generated by using the RIP (15, 16). In our model, the animals do not spontaneously become diabetic. However, after infection with LCMV-WE insulitis involving CD4$^+$ but predominantly CD8$^+$ T cells is apparent and approximately 11 days later the mice become hyperglycemic. Because the self Ag that is the target of immunopathologic attack is known, this model becomes amenable to detailed analysis. Various viruses exist that express the LCMV-gp, and low and high responder mouse strains have been defined for the LCMV-gp Ag. Two compatible transgenic systems are available, one expressing TNF-α under the control of the RIP (17) and another in which virtually all CD8$^+$ T cells express a TCR specific for the LCMV-gp (18). By using various viruses combined with the transgenic models available, this study suggests that the MHC haplotype, the level of MHC expression, the infectious agent, TNF-α expression, and the number of activated self-reactive CTL are important factors in determining the development of overt autoimmune disease. These findings also have relevant implications regarding peripheral tolerance induction.

[6] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; gp, glycoprotein; vacc, vaccinia virus; RIP, rat insulin promoter.

Materials and Methods

Animals

Transgenic animals were housed under conventional conditions (Universitatsspital, Zurich, Switzerland), and mice were bred and maintained with the C57BL/6 inbred strain according to federal, kantonal, and institutional regulations. Two different lines of RIP-gp (H-2^b)-transgenic animals have been reported, Bln and Brx (15). For certain experiments, the transgenic mice were bred with B10.BR (H-2^k) animals. Transgenic F_1 animals were backcrossed to B10.BR and typed for the H-2^b allele by using mAb B8–24–3 (19). This antibody reacts with H-2K^b and does not cross-react with H-2^k. Those animals that did not express H-2^b and were positive for the gp transgene were used to breed with B10.BR animals.

The RIP-TNF-α animals have been described (17). Two lines of TCR-transgenic animals were used, 327 and 318. Both lines express the transgenic TCR specific for LCMV-gp peptide 32–42 presented in the context of H-2D^b (18). The 327 line expresses the transgenic receptor on approximately 90% of the CD8$^+$ T cells (20), whereas the 318 line expresses the transgenic receptor on approximately 50% of the CD8$^+$ T cells (Table I).

Typing of transgenic animals

DNA was isolated from tail biopsies by mincing the tail and incubating it in proteinase K (Merck) at 55°C overnight (50 mM Tris, pH 7.6, 100 mM EDTA, pH 8.0, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K). The mixture was centrifuged for 2 min to pellet the debris and the aqueous phase was extracted once with phenol/chloroform. The DNA was precipitated in isopropanol and 0.2 μg was used in a polymerase chain reaction. The primers used to detect the gp transgene were 5′-CAAGCAAGATGTAGAGTCTGCC and 5′-GGCTTTGGACATGAACCGCCC, to detect the TCR α-chain were 5′-CGAGGATCCTTTAACTGGTA-CACAGCAGG and 5′-CTGACCTGCAGTTATGAGG-ACAGCAC, and to identify RIP-TNF-α-transgenic mice were 5′-TAAGGCTAAGTAGAGGTGT and 5′-GAGAA-GAGGCTGAGACATAG.

Viruses

Several different viruses have been used in this study, as follows: LCMV-WE (21), LCMV-Armstrong (21), LCMV-Pasteur (22), LCMV-8.7 (20), LCMV-clone 13 (23), and vacc-gp (24) (vacc-G2). Wild-type vacc Westerm Reserve and the tyrosine kinase-negative variant were kind gifts from D. H. L. Bishop, Oxford University (Oxford, UK) and from B. Moss, National Institutes of Health (Bethesda, MD), respectively. Single-transgenic animals were infected i.v. with 200 PFU of all LCMV strains. For recombinant vacc infections, 2×10^6 PFU of vacc-gp were routinely given, because this dose is required to induce a measurable vacc-specific CTL response (24). Double-transgenic animals (RIP-gp/TCR) were given 2×10^6 PFU of LCMV-WE. The TCR-transgenic mice have a high precursor frequency of LCMV-gp-specific T cells and consequently they are able to quickly mount a strong response against a high dose of the virus (20).

Blood glucose measurements were determined by using a Haemo-glucotest kit and were quantitated with Reflolux II (Boehringer, Mannheim, Germany). Animals were considered diabetic when blood glucose levels persisted above 14 mM for at least 7 days.

T cell depletion in vivo

Transgenic and control animals were depleted of either the $CD4^+$ or $CD8^+$ T cell subset by treatment in vivo with mAb. Animals were given 0.2 ml (0.5 mg) of either anti-CD4 (YTS191.1) or anti-CD8 (YTS169.4.2) mAb (25) i.v., 3 days and 1 day before virus infection.

Immunohistochemistry

Pancreata were immersed in HBSS and snap frozen in liquid nitrogen. Cryostat sections of tissue 5-μm thick were cut and fixed in acetone for 10 min. Sections were incubated with the primary antibodies YTS169.4.2 (anti-CD8) (25), YTS191.1 (anti-CD4) (25), F4/80 (anti-macrophage) (26), M1/42 (anti-class I MHC) (27), M5/114 (anti-class II) (28), or RA3 6B2 (anti-CD45, B cell marker) (29). Primary antibodies were followed by a two-step indirect immunoenzymatic staining procedure. Alkaline phosphatase-labeled goat anti-rat Ig (TAGO, Burlingame, CA) was added for 30 min at room temperature, followed by alkaline phosphatase-labeled anti-goat antibodies (Jackson Immunoresearch, PA) for another 30 min. Dilutions were prepared in 0.1 M Tris-HCl (pH 7.4) containing 5% normal mouse serum. Alkaline phosphatase was then detected by a red color reaction, by using naphthol A-BI phosphate and New Fuchsin. Endogenous alkaline phosphatase was blocked with levamisol. Sections were counterstained with Mayer's hemalum for 2 min.

The amount of infiltration in the islets of Langerhans was scored on an arbitrary scale upon examination of approximately 20 islet sections (–, no positive cells detected; +, <10 cells; ++, 10 to 20 cells; +++, >20 cells).

Table II
Effect of MHC haplotype on the onset of diabetes

Mice	Haplotype	No. of Diabetic Mice	Onset of Diabetes (day)[a]
C57BL/6	b	0/2	
RIP-gp(Bln) C57BL/6	b	8/8	10.9 ± 0.4
B10.BR	k	0/2	
RIP-gp(Bln) B10.BR	k	5/6	23.8 ± 3.7[b]

[a] Day (mean ± SEM) of onset of hyperglycemia above 20 mM.
[b] Does not include the animal that became hyperglycemic on day 60 or the animal that did not develop diabetes.

Results

Effect of MHC haplotype on the onset of diabetes

Analysis has shown that the MHC of an individual may be correlated with a predisposition to certain autoimmune diseases (for reviews, see Refs. 1, 30, and 31). The effect of MHC haplotype on the LCMV-gp response has been reported previously; $H\text{-}2^k$ animals show a poor cytotoxic response to the gp, compared with the $H\text{-}2^b$-restricted response (24). Therefore, to test the effect of MHC haplotype on diabetes in our system, the RIP-gp(Bln) transgenic line was bred with the B10.BR ($H\text{-}2^k$) strain. $H\text{-}2^{b/k}$ RIP-gp-(Bln) transgenic animals and their nontransgenic littermates were infected with 200 PFU of LCMV-WE. Hyperglycemia was detected on approximately day 24 in four of six $H\text{-}2^k$ transgenic animals (Table II). Another animal required 60 days after infection to become diabetic. The sixth animal did not become diabetic over the observed time period of 4 months. Therefore, although mice with the $H\text{-}2^k$ haplotype responded poorly to LCMV-gp, some gp-specific T cell reactivity was induced and diabetes still occurred, with altered kinetics.

Role of Th cells in inducing diabetes

The importance of $CD4^+$ T cells, in addition to the essential role of $CD8^+$ T cells, was determined by treating the animals with anti-CD4 or anti-CD8 antibodies in vivo. For $H\text{-}2^b$ RIP-gp-transgenic animals, two different RIP-gp lines, Bln and Brx, were further analyzed. Results showed that independent treatment with either anti-CD4 or anti-CD8 could block the onset of diabetes in the Brx line, as reported previously (15), whereas only anti-CD8 could block diabetes in the Bln line (Table III). The explanation for why these two lines have different requirements for the CD4 subset in the induction of diabetes is not known. Both transgenic lines were developed in the C57BL/6 inbred strain, and both have the same kinetics of diabetes development and the same infiltrating cells in the islets.

Table III
T cell subsets required for onset of diabetes vary with MHC haplotype

Transgenic line	Haplotype	No. of Mice with Diabetes after Treatment with	
		Anti-CD4	Anti-CD8
RIP-gp(Brx)	b	0/4	0/4
RIP-gp(Bln)	b	4/4	0/4
RIP-gp(Bln)	k	0/6	0/6

The Bln line was used to study the role of $CD4^+$ T cells in the $H\text{-}2^k$ haplotype. In vivo antibody treatment showed that either anti-CD4 or anti-CD8 could block diabetes in $H\text{-}2^k$ mice, whereas only anti-CD8 could block diabetes in the $H\text{-}2^b$ Bln mice (Table III). Therefore, whereas the $CD8^+$ T cells were an absolute prerequisite for the induction of diabetes, the $CD4^+$ T cells were required in $H\text{-}2^k$ but not $H\text{-}2^b$ transgenic Bln animals. This suggests that $CD4^+$ T cells may be necessary in CD8-low responder situations. These studies with the RIP-gp(Bln) transgenic animals indicate that the MHC haplotype affects the induction of diabetes in at least two ways; it determines the amount of T help necessary and influences the kinetics of disease.

Immunohistochemical analysis of the cells present during the induction of diabetes

As reported previously, 8 days after LCMV infection the lymphocyte infiltrate in the islets included both $CD4^+$ and predominantly $CD8^+$ cells. This insulitis was found only in RIP-gp-transgenic animals and not in the nontransgenic littermate controls (15). To determine whether other cells are recruited to the pancreas, whether the cells are recruited in a certain order, and whether the levels of class I and class II MHC expression are important, immunohistochemical analysis was performed. C57BL/6 and RIP-gp(Bln) animals were infected with 200 PFU of LCMV-WE and two transgenic and control animals were sacrificed on alternate days, up to 16 days after virus infection (Table IV; Fig. 1). Class I expression was increased on all islet cells 2 days after LCMV infection in both C57BL/6 control animals and transgenic animals (Fig. 1). Class I expression was further increased from approximately day 6 and was slightly higher in transgenic animals, probably because of the staining of the infiltrating cells. Class II^+ cells remained scattered randomly throughout the pancreas after infection with LCMV in C57BL/6 animals. An increase in class II^+ cells was seen from day 8 to day 12 after LCMV infection in transgenic mice, but again this was likely the result of the infiltrating cells. Macrophages were detectable 4 days after LCMV infection and persisted throughout the monitored period. Slightly more macrophages were scattered through the islets of transgenic animals approximately 8 days after LCMV infection, but the majority were located around the islets. Very few B cells were detected in the islets of transgenic animals 8 days after LCMV infection, and they were not detected in every islet (Fig. 1). Both peri-insular and infiltrating $CD4^+$ T cells, as well as a predominant $CD8^+$ population, were found in transgenic animals beginning 8 days after infection with LCMV but remained few or undetectable in control C57BL/6 animals. Pancreas sections taken after day 12 exhibited a drastically disrupted islet morphology, coinciding with hyperglycemia.

Induction of diabetes by using various viruses expressing the LCMV-gp Ag

RIP-gp(Bln) animals were infected with various viruses expressing the LCMV-gp Ag. Natural variants of LCMV were used (Armstrong, Pasteur, and clone 13) as well as T cell epitope escape mutants (8.7) and vacc recombinants expressing the LCMV-gp (vacc-gp) (Table V). The Armstong, Pasteur, clone 13, and 8.7 strains of LCMV were able to induce diabetes with similar kinetics after infection with 200 PFU.

RIP-gp-transgenic mice were also infected with 2×10^6 PFU of recombinant vacc expressing the LCMV-gp (vacc-gp), but interestingly these animals did not develop diabetes (Table V). Multiple injections of vacc-gp were given to RIP-gp-transgenic animals in an attempt to induce diabetes (4×10^6 PFU, for 3 consecutive days); however, none of the animals showed an increase in nonfasting glucose levels. These results suggest that the properties of the infectious virus may determine whether diabetes occurs in this model.

Induction of diabetes by vacc-gp in RIP-gp/TCR-transgenic mice

The failure of vacc-gp to induce diabetes in the RIP-gp-transgenic Bln or Brx mice suggested that there might be a quantitative threshold for induction of disease. Previous studies showed that when C57BL/6 animals were immunized with vacc-gp (2×10^6 PFU) a cytotoxic T cell response specific for LCMV-gp could not be detected, despite a strong vacc-specific CTL response (as measured in a cytotoxicity assay 6 days after vacc-gp infection). However, a memory CTL response against the LCMV-gp Ag could be measured in vitro or in vivo after priming with vacc-gp (24). Thus, relatively few LCMV-gp-specific CTL are activated after infection of normal mice with vacc-gp. It is possible that the gp-specific CTL response induced after vacc-gp infection may be insufficient to cause immunopathologic damage resulting in diabetes in RIP-gp-transgenic mice.

To address the question of whether the onset of diabetes is related to the number of responding gp-specific CTL, double-RIP-gp/TCR-transgenic mice (Brx/327) expressing the transgenic TCR specific for LCMV-gp plus $H\text{-}2D^b$

Table IV
Histologic analysis of islets after LCMV infection

	Nontransgenic C57BL/6/RIP-gp(Bln)					
	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Class I[a]	+/+[b]	+/+	++/++	++/+++	+++/+++	++/+++
Class II[c]	+/+	+/+	+/+	+/+++	+/+++	+/+++
Macrophages	−/−	+/+	+/+	+/++	+/+	+/+
B cells	−/−	−/−	−/−	−/+	−/+	−/+
CD4	−/−	−/−	−/−	−/++	−/++	−/++
CD8	−/−	−/−	−/−	−/+++	−/+++	−/+++

[a] Uniform positive staining.
[b] Arbitrary scale (see *Materials and Methods*).
[c] Scattered positive cells.

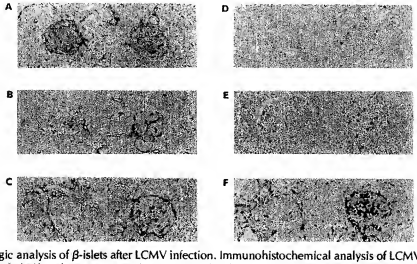

FIGURE 1. Histologic analysis of β-islets after LCMV infection. Immunohistochemical analysis of LCMV-infected RIP-gp(Bln) transgenic animals (*right half*) and nontransgenic littermates (*left half*) with antibodies specific for class I MHC (*A*), class II MHC (*B*), macrophages (*C*), B cells (*D*), CD4 (*E*), and CD8 (*F*). *A* and *B*, 2 days after LCMV infection; *C* to *F*, 8 days after LCMV infection.

(327) (18) were infected with 2×10^6 PFU of vacc-gp. These animals showed an increase in blood glucose levels 4 days after infection (Fig. 2). Control RIP-gp/TCR-transgenic animals infected with wild-type vacc did not develop diabetes (Table V).

To investigate the importance of the frequency of gp-specific CTL, we took advantage of another TCR-transgenic line, termed 318. This line expresses the gp-specific transgenic TCR on approximately 50% of $CD8^+$ T cells, whereas the 327 line expresses the transgenic TCR on 90 to 95% of $CD8^+$ T cells (20). Double-RIP-gp/TCR-transgenic mice (Brx/318) were infected with 2×10^6 vacc-gp, and these animals showed an increased glucose level after 8 days (Fig. 2). Surprisingly, an approximately two-fold reduction in gp-specific CTL altered the kinetics of hyperglycemia when these animals were infected with vacc-gp. Thus, these results indicate that the frequency of responding gp-specific T cells influences the agents that are able to induce disease and may consequently alter the kinetics of immunopathologic disease.

Levels of class I MHC expression in infected gp-trangenic animals

Because single-RIP-gp-transgenic animals did not become diabetic upon infection with vacc-gp, histologic analysis was performed. Six days after infection with vacc-gp, both $CD4^+$ and $CD8^+$ infiltrates were seen in the pancreata of RIP-gp(Bln) transgenic mice only and not in C57BL/6 controls. However, unlike histologic sections from RIP-gp-transgenic mice infected with LCMV-WE virus, where all islets were infiltrated (15), lymphocytes were detected in only a few islets after vacc-gp infection (data not shown).

Because histologic analysis showed that the level of MHC Ag increased upon LCMV infection (see above and Fig. 1), the pancreata of animals infected with vacc-gp were also examined to evaluate the importance of MHC up-regulation in the progression of disease. Immunohistochemical analysis showed that in general the MHC levels were not increased in C57BL/6 animals infected with vacc-gp 2, 4, 6, and 8 days after viral infection (Table VI). How-

Table V
Induction of diabetes by using viruses expressing LCMV-gp

Immunizing Agent	Mice (Line and Haplotype)	No. of Diabetic Mice	Onset of Diabetes (day) [a]
LCMV-WE (200 PFU)	RIP-gp(Bln) (b)	8/8	8, 9, 9, 10, 10, 11, 12, 13
LCMV-Armstrong (200 PFU)	RIP-gp(Bln) (b)	2/2	9, 9
LCMV-Pasteur (200 PFU)	RIP-gp(Bln) (b)	4/4	11, 11, 11, 11
LCMV-clone 13 (200 PFU)	RIP-gp(Bln) (b)	2/2	11, 11
LCMV-8.7 (200 PFU)	RIP-gp(Bln) (b)	2/2	13, 13
vacc-gp (2×10^6 PFU)	RIP-gp(Bln) (b)	0/6	
vacc-gp (2×10^6 PFU)	RIP-gp(Bln)/TNF (b)	2/4	7, 10
vacc-gp (2×10^6 PFU)	C57BL/6 (b)	0/4	
Wild-type vacc (2×10^6 PFU)	RIP-gp/TCR(Bln/327) (b)	0/2	
No treatment	RIP-gp(Bln) (b)	0/191	

[a] Day of onset of hyperglycemia above 20 mM.

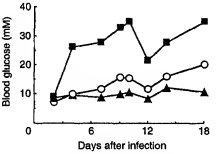

FIGURE 2. Effect of the infectious virus and T cell precursor frequencies on the onset of diabetes. RIP-gp(Brx) animals (▲), RIP-gp/TCR(Brx/318) animals (O) expressing the transgenic TCR on approximately 50% of the $CD8^+$ T cells, and RIP-gp/TCR(Brx/327) animals (■) expressing the transgenic TCR on approximately 90% of $CD8^+$ T cells were challenged with 2×10^6 PFU of vacc-gp i.v. The blood glucose concentration (mM) was measured at various time points after infection. These data are representative of two animals from each group.

Table VI
Increase in MHC class I corresponds to the onset of diabetes

Mice	Infectious Agent	MHC Levels [a]	Diabetes
C57BL/6	None	Low	–
C57BL/6	LCMV	High	–
C57BL/6	vacc-gp	Low	–
RIP-gp	None	Low	–
RIP-gp	LCMV	High	+
RIP-gp	vacc-gp	Low	–
TCR	None	Low	–
RIP-gp/TCR	None	Low	–
RIP-gp/TCR	LCMV	High	+
RIP-gp/TCR	vacc-gp	High	+

[a] Class I MHC expression on β-Islet cells.

ever, in some instances slight increases in MHC class I levels were detected (Fig. 3). It is possible that $CD4^+$ and $CD8^+$ T cells were able to recognize the gp Ag on the surface of those few islets that had up-regulated MHC expression. However, this was not sufficient to induce overt hyperglycemia, because infiltration and immunopathology did not occur in every islet.

If MHC up-regulation is important for gp self Ag expression and is required for efficient CTL recognition, then the double-RIP-gp/TCR-transgenic mice that were infected with vacc-gp should also show an increase in MHC expression. Because RIP-gp/TCR-transgenic mice developed diabetes 3 to 4 days after virus infection, the pancreata of these double-transgenic animals were examined 24 h after infection. Interestingly, these animals clearly showed that the levels of class I were increased in the islets of Langerhans (Fig. 3; Table VI) before $CD4^+$ or $CD8^+$ cells infiltrated the islets. Taken together, these findings emphasize the importance of up-regulation of class I expression on the islets of Langerhans in the progression and development of autoimmune disease in this model.

Evidence that increased lymphocyte traffic in the islets leads to diabetes in RIP-gp-transgenic mice infected with vacc-gp

The studies examining the level of MHC class I expression after vacc-gp infection suggest that diabetes may not occur in the transgenic animals because the gp-specific CTL do not find their way to the islets. Recently, TNF-α-transgenic animals have been generated by using the RIP; analysis has shown that the islets are heavily infiltrated but these animals do not develop diabetes (17). Double-transgenic mice expressing both LCMV-gp and TNF-α under the control of the RIP were infected with vacc-gp to determine whether the combination of increased lymphocyte traffic and the activation of few gp-specific CTL could induce diabetes. Notably, two of four double-transgenic animals became acutely hyperglycemic (Table V). The MHC class I levels in these animals were also examined and shown to be very high. However, it is difficult to determine whether high levels of class I MHC are expressed on both the islet cells and the infiltrating lymphocytes.

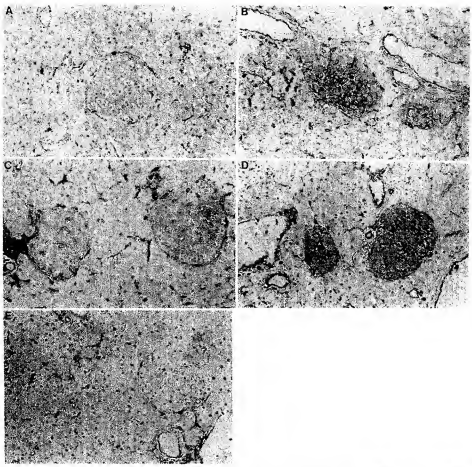

FIGURE 3. Levels of MHC class I expression on islet cells from transgenic animals infected with different viruses expressing LCMV-gp. Immunohistochemical analysis was performed with class I-specific mAb on pancreata from uninfected C57BL/6 control mice (*A*), C57BL/6 mice 8 days after LCMV-WE infection (*B*), C57BL/6 mice 2 days after vacc-gp infection (selected islets expressing detectable class I; the majority of islets showed very low class I expression 2, 4, 6, and 8 days after vacc-gp infection) (*C*), RIP-gp/TCR-transgenic mice 1 day after vacc-gp infection (in serial sections, $CD4^+$ and $CD8^+$ T cells were seen around but not infiltrating the islets) (*D*), and uninfected RIP-gp/TCR-transgenic animals (*E*), which were the same as uninfected C57BL/6 animals.

Discussion

Transgenic animals that express the LCMV-gp in the islet cells of the pancreas have been used to evaluate the effect of MHC haplotype on the onset of diabetes. The RIP-gp transgene was bred into the H-2^k haplotype, which is known to generate a poor cytotoxic response specific for the LCMV-gp (24). Hyperglycemia was detected approximately 24 days after infection in H-2^k RIP-gp-transgenic animals infected with LCMV, whereas hyperglycemia was seen approximately 11 days after infection in H-2^b RIP-gp-transgenic animals. The $CD4^+$ subset was required for induction of diabetes in H-2^k RIP-gp(Bln) animals but not in H-2^b RIP-gp(Bln) animals. Histologic analysis of infected H-2^k RIP-gp(Bln) transgenic mice showed a predominantly $CD4^+$ lymphocytic infiltrate (data not shown). Therefore, the predominance of $CD4^+$ or $CD8^+$ cells in infiltrated organs may depend upon the "strength" of the immune response to a given self Ag. These studies demonstrate that the class I MHC haplotype affects the kinetics of disease and the requirement for the $CD4^+$ subset.

Experiments with this viral transgenic model have illustrated that the properties of the infectious agent bearing a self Ag may alter the onset of diabetes. In this model, diabetes is induced after infection of the RIP-gp-transgenic animals with various strains of LCMV. Histologic analysis has shown that $CD4^+$ cells, $CD8^+$ cells, and macrophages are present in the islets of LCMV-infected animals, and an increase of class I MHC is apparent. However, hyperglycemia does not occur when the RIP-gp-transgenic animals are infected with vacc-gp, which does not generate a strong gp-specific CTL response and which also is not efficient in up-regulating class I expression on the pancreatic islet cells in C57BL/6 mice. When double-RIP-gp/TCR-transgenic mice are immunized with vacc-gp, the large number of

transgenic gp-specific T cells are able to respond, class I expression is increased on the islet cells (because of cytokine production from the gp-specific transgenic T cells), and overt hyperglycemia ensues.

Thus, although vacc-gp has the potential to induce autoreactive T cells, hyperglycemia does not occur in RIP-gp animals that posses a "normal" unbiased T cell repertoire. This suggests that activation of sufficient numbers of autoreactive T cells that could result in detectable autoimmune disease may be difficult in some circumstances. Induction of CD8-dependent autoimmunity may rely upon a "unique" virus that not only possesses an antigen that is immunologically identical to the self molecule but possesses an epitope that can activate a strong immune response specific for the virus and a self molecule. Examples such as these may be one of many factors that determine why the presence of self-reactive cells does not lead to widespread autoimmunity.

Studies with the double-RIP-gp/TNF-α-transgenic mice also suggest that a combination of gp-specific T cell activation and increased lymphocyte traffic in the islets of Langerhans may enhance an autoaggressive immune response enough to result in hyperglycemia. The role for TNF-α in autoimmune responses is controversial (32–34). Our observations are consistent with the idea that the few gp-specific T cells activated by vacc-gp infection are able to interact with the Ag on the islets because the expression of TNF-α increases the local lymphocyte traffic. Previous studies showed that diabetes did not occur in RIP-TNF-transgenic animals infused with IFN-γ. Although increased MHC class I expression and increased lymphocyte traffic were observed in the islets of Langerhans, diabetes may not have occured because islet Ag-specific T cells were not sufficiently activated by appropriate stimuli or "professional APC." Other data also support this interpretation. Infusion of IFN-γ alone did not result in hyperglycemia in the RIP-gp-transgenic line, suggesting that the specific activation of self-reactive T cells was necessary for induction of disease (15). Allison et al. (35) expressed IL-2 in the islets of Langerhans and hyperglycemia was not detected, despite the presence of infiltrating cells. It is possible that an islet-specific immune response was not activated in this model and therefore autoimmune disease was not detected.

In summary, these data suggest that the infectious agent is important in determining the immunopathologic response in RIP-gp-transgenic animals and that the efficient induction of gp-specific CTL and an increase in class I gene expression on the islet cells are important for the establishment of diabetes in this model. It is also likely that other parameters play a role in influencing the development of diabetes, including cytokines and adhesion and lymphocyte-trafficking molecules.

The results from the studies with RIP-gp-transgenic animals infected with vacc-gp provide interesting insights into the requirements for CTL memory and maintenance of effector function in vivo. As we have demonstrated, vacc-gp-infected RIP-gp-transgenic animals do not become diabetic over a period of 3 months. gp-specific T cells have been demonstrated in vacc-gp-primed animals (24), but these cells apparently are not perpetually stimulated by the gp expressed on the islet cells and cumulative damage resulting in diabetes does not occur. This finding is relevant in at least two important situations. With respect to induction of CTL-mediated autoimmune disease, these results suggest that a strong primary CTL response is sufficient but the existence of "memory" CTL in the presence of the gp Ag on the islets is unable to cause sufficient immunopathologic damage to result in diabetes. These findings also have implications for potential immunotherapy against tumors. In the case where tumor growth may be controlled by CTL, our model would predict that, even if an individual were vaccinated against a tumor-specific Ag, the effector CTL function alone may not be sufficient to control tumor growth.

The results of these studies also have implications for peripheral tolerance models and peripheral T cell tolerance in general. Our data imply that the CTL responsible for mediating this disease require an increase in class I gene expression and thus a corresponding increase in self Ag presentation on the β-islet cells. Although the islets have been shown to express class I MHC (36, 37), the levels may be below a threshold required for CTL recognition, or expression of a given self Ag may be too low for tolerance induction to all self Ag. This may be an important parameter that distinguishes this model, where self-reactive peripheral T cells ignore the self Ag, from other models of peripheral tolerance. Other studies with transgenic MHC self Ag have shown that T cells specific for the transgenic MHC Ag have become tolerized in a variety of ways (7, 8, 38–42). This T cell tolerance may have been necessary because the level of transgenic MHC Ag on the surface could be considerably higher than the level of self MHC complexed with a nominal Ag. Therefore, it remains possible that differences in the levels of MHC and self Ag presentation may be crucial in determining the state of peripheral T cell nonreactivity to defined self Ag.

Acknowledgments

We would like to thank Alana Althage for assistance in transgenic analysis, Dow-Rhoon Koh, Klaus Pfeffer, and Marco Schilham for critical reading of the manuscript, and Ms. Marissa Luchico for preparation of the manuscript.

References

1. Sinha, A. A., M. T. Lopez, and H. O. McDevitt. 1990. Autoimmune diseases: the failure of self tolerance. *Science 248:1380.*
2. Castano, L., and G. S. Eisenbarth. 1990. Type-I diabetes: a

chronic autoimmune disease of human, mouse, and rat. *Annu. Rev. Immunol. 8:647.*

3. Zamvil, S. S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579.*
4. Hanahan, D. 1990. Transgenic mouse models of self-tolerance and autoreactivity by the immune system. *Annu. Rev. Cell Biol. 6:493.*
5. Ramsdell, F., and B. J. Fowlkes. 1990. Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. *Science 248:1342.*
6. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell 63:1249.*
7. Schoenrich, G., U. Kalinke, F. Momburg, M. Malissen, A. M. Schmitt-Verhulst, B. Malissen, G. J. Hämmerling, and B. Arnold. 1990. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell 65:293.*
8. Schoenrich, G., F. Momburg, M. Malissen, A. M. Schmitt-Verhulst, B. Malissen, G. Hammerling, and B. Arnold. 1992. Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self antigen. *Int. Immunol. 4:581.*
9. Lo, D., J. Freedman, S. Hesse, R. D. Palmiter, R. L. Brinster, and L. A. Sherman. 1992. Peripheral tolerance to an islet cell-specific hemagglutinin transgene affects both $CD4^+$ and $CD8^+$ T cells. *Eur. J. Immunol. 22:1013.*
10. Boehme, J., K. Haskins, P. Stecha, W. van Ewijk, M. LeMeur, P. Gerlinger, C. Benoist, and D. Mathis. 1989. Transgenic mice with I-A on islet cells are normoglycemic but immunologically intolerant. *Science 244:1179.*
11. Murphy, K. M., C. T. Weaver, M. Elish, P. M. Allen, and D. Y. Loh. 1989. Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: evidence against a clonal-deletion mechanism. *Proc. Natl. Acad. Sci. USA 86:10034.*
12. Miller, J., L. Daitch, S. Rath, and E. Selsing. 1990. Tissue-specific expression of allogeneic class II MHC molecules induces neither tissue rejection nor clonal inactivation of alloreactive T cells. *J. Immunol. 144:334.*
13. Götz, J., H. Eibel, and G. Köhler. 1990. Non-tolerance and differential susceptibility to diabetes in transgenic mice expressing major histocompatibility class II genes on pancreatic beta cells. *Eur. J. Immunol. 20:1677.*
14. Schild, H. J., O. Rötzschke, H. Kalbacher, and H.-G. Rammensee. 1990. Limit of T cell tolerance to self proteins by peptide presentation. *Science 247:1587.*
15. Ohashi, P. S., S. Oehen, K. Bürki, H. P. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell 65:305.*
16. Oldstone, M. B. A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell 65:319.*
17. Herrera, P., Y. Higuchi, P. Muniesa, J. Huarte, D. Belin, P. S. Ohashi, P. Aichele, L. Orci, J. D. Vassalli, and P. Vassalli. 1992. Expression of a TNF-alpha transgene in murine pancreatic beta cells results in severe and permanent insulitis without evolution towards diabetes. *J. Exp. Med. 176:1719.*
18. Pircher, H. P., K. Bürki, R. Lang, H. Hengartner, and R. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature 342:559.*
19. Köhler, G., K. Fischer-Lindahl, and C. Heusser. 1981. Characterization of a monoclonal anti-H-$2K^b$ antibody. In *The Immune System*, 2nd Ed. C. Steinberg and I. Lefkovits, eds. Karger, Basel, p. 202.
20. Pircher, H. P., D. Moskophidis, U. Rohrer, K. Bürki, H. Hengartner, and R. M. Zinkernagel. 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature 346:629.*
21. Lehmann-Grube, F. 1971. Lymphocytic choriomeningitis virus. *Virol. Monogr. 10:1.*
22. Saron, M. F., B. Shidani, M. A. Nahori, J. C. Guillon, and P. Truffa Bachi. 1990. Lymphocytic choriomeningitis virus-induced immunodepression: inherent defect of B and T lymphocytes. *J. Virol. 64:4076.*
23. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med. 60:521.*
24. Hany, M., S. Oehen, M. Schulz, H. Hengartner, M. Mackett, D. H. L. Bishop, and R. M. Zinkernagel. 1989. Anti-viral protection and prevention of lymphocytic choriomeningitis or of the local footpad swelling reaction in mice by immunisation with vaccinia-recombinant virus expressing LCMV-WE nucleoprotein or glycoprotein. *Eur. J. Immunol. 19:417.*
25. Cobbold, S. P., A. Jayasuriya, A. Nash, T. D. Prospero, and H. Waldmann. 1984. Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. *Nature 312:548.*
26. Austyn, J. M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol. 11:805.*
27. Springer, T. A. 1980. Cell-surface differentiation in the mouse. In *Monoclonal Antibodies*. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, eds. Plenum Press, New York, p. 185.
28. Bhattacharya, A., M. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol. 127:2488.*
29. Coffman, R. L. 1982. Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol. Rev. 69:5.*
30. Todd, J. A., H. Acha-Orbea, J. I. Bell, N. Chao, Z. Fronek, C. O. Jacob, L. Timmermann, L. Steinmann, and H. O. McDevitt. 1988. A molecular basis for MHC class 2-associated autoimmunity. *Science 240:1003.*
31. Nepom, G. T., and H. Erlich. 1991. MHC class II molecules and autoimmunity. *Annu. Rev. Immunol. 9:493.*
32. Jacob, C. O. 1992. Tumor necrosis factor alpha in autoimmunity: pretty girl or old witch? *Immunol. Today 13:122.*
33. Lane, J. R., D. A. Neumann, A. Lafond-Walker, A. Herskowitz, and N. R. Rose. 1992. Interleukin 1 or tumor necrosis factor can promote coxsackie B3-induced myocarditis in resistant B10.A mice. *J. Exp. Med. 175:1123.*

34. Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol. 10:411.*
35. Allison, J., L. Malcolm, N. Chosich, and J. F. A. P. Miller. 1992. Inflammation but not autoimmunity occurs in transgenic mice expressing constitutive levels of interleukin-2 in islet β cells. *Eur. J. Immunol. 22:1115.*
36. Faustman, D., V. Hauptfeld, J. M. Davie, P. E. Lacy, and D. C. Shreffler. 1980. Murine pancreatic beta-cells express H-2^K and H-2^D but not Ia antigens. *J. Exp. Med. 151:1563.*
37. Baekkeskov, S., T. Kanatsuna, L. Klareskog, D. A. Nielsen, P. A. Peterson, A. H. Rubenstein, D. F. Steiner, and A. Lernmark. 1981. Expression of major histocompatibility antigens on pancreatic islet cells. *Proc. Natl. Acad. Sci. USA 78:6456.*
38. Fields, L. E., and D. Y. Loh. 1992. Organ injury associated with extrathymic induction of immune tolerance in doubly transgenic mice. *Proc. Natl. Acad. Sci. USA 89:5730.*
39. Morahan, G., F. E. Brennan, P. S. Bhathal, J. Allison, K. O. Cox, and J. F. A. P. Miller. 1989. Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter. *Proc. Natl. Acad. Sci. USA 86:3782.*
40. Morahan, G., J. Allison, and J. F. A. P. Miller. 1989. Tolerance of class I histocompatibility antigens expressed extrathymically. *Nature 339:622.*
41. Burkly, L. C., D. Lo, O. Kanagawa, R. L. Brinster, and R. A. Flavell. 1989. T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature 342:564.*
42. Wieties, K., R. E. Hammer, S. Jones-Youngblood, and J. Forman. 1990. Peripheral tolerance in mice expressing a liver-specific class I molecule: inactivation/deletion of a T-cell subpopulation. *Proc. Natl. Acad. Sci. USA 87:6604.*